IDENTIFICATION OF A UMAMI PEPTIDE IN TEMPEH (INDONESIAN FERMENTED FOOD) BY LC-MS/MS AND THE BINDING MECHANISM TO THE UMAMI RECEPTOR

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經本委員會審定通過，特此證明。

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PERNYATAAN
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ABSTRACT

Tempeh is the soybean product, which produced by fermentation. It was known in the 1700s in Indonesia, and first made in Java, Indonesia. The tempeh household consumption percentage in year 2009 was 69.89%, much more than other soy based products, such as tofu, sauce, oncom, tauco and soymilk. During fermentation, protein digestion occurs, caused by proteases secreted during the growth of *Rhizopus microsporus var. oligosporus*. The soy protein is digested to form free amino acids and peptides. Tempeh is especially popular because of its umami taste, which may be caused by small peptide compositions of size range 1-3 kDa containing some Glu and Asp sequences. The umami (T1R) receptor has two subtypes, T1R1 and T1R3, which can function as heterodimers or homodimers. In this study, our aims were to identify peptides that contribute to the umami taste, using LC-MS/MS, sensory evaluation and molecular docking to the umami receptor. The water soluble extracts from tempeh were fractionated using a 3 kDa molecular weight cutoff ultrafiltration membrane and the taste profile of the low molecular weight fraction was evaluated by six panelists who were selected from Department of Biological Science and Technology, National Pingtung University of Science and Technology, Taiwan. The panelists were trained to recognize the five basic tastes using some standard solutions for taste agents including sweet, bitter, sour, salty and umami represented by solutions of sucrose (1%), caffeine (0.08%), citric acid (0.08%), sodium chloride (0.35%), and MSG–salt (0.35–0.35%), respectively. Then, the panelists tested the five basic tastes for (a) water soluble extract from tempeh as well the standard solution; (b) the difference test for five basic tastes between water soluble extract and < 3 kDa cutoff fraction; and (c) the difference test between < 3 kDa cutoff fraction and synthetic peptide GENEEEDSGAIVTVK . The results showed that umami had the highest intensity compared to the other basic tastes in < 3 kDa cutoff fraction and synthetic peptide GENEEEDSGAIVTVK. LC-MS/MS identified 4 peptides from the < 3 kDa cutoff fraction of tempeh at 48 h, including GENEEEDSGAIVTVK, which we synthesized and evaluated. This peptide intensified umami taste, although the umami peptide intensity is not significantly different from the < 3 kDa cutoff fraction. The results of molecular docking using ZDOCK showed that the peptide could bind to the binding pocket of TIR3 in open and closed conformations.

Keywords : Tempeh, umami, peptide, T1R, docking

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I. Introduction

1.1. Background

Tempeh is the soybean product, which produced by fermentation (Baumann & Bisping, 1995). Tempeh was known in the 1700s in Indonesia, and first made in Java, Indonesia. The tempeh household consumption precentage in year 2009 was 69.89 %, compared with other soy based products, such as tofu, sauce, oncom, tauco and soymilk. Other countries, that was identified with tempeh consumer are China, Taiwan, Australia, Japan, some countries in Europe, America and Africa. The competitive advantages of tempeh have been discovered, such as high content of crude protein, isoflavone, vitamine B12, folate, fat, and carbohydrate (Mo, Kariluoto, Piironen, Zhu, Sanders, Vincken, et al., 2013) the activity of antihypertention due to its small peptide composition (Gibbs, Zougman, Masse, & Mulligan, 2004) and the umami taste. Those competitive advantages of tempeh may affect the high consumption percentage of some countries.

Tempeh is produced by following processes, whole soybean is acidified, boiled, cooled, and inoculated with *Rhizopus microsporus var. oligosporus*, it grows troughput boiled soybeans and transform the soybean into a compatc cake (tempeh). The protein digestion could be occured to fermentation, because the prodution of protease during the growth of *Rhizopus microsporus var. oligosporus. Rhizopus microsporus var. oligosporus* belonging to the zygomycete class, also secretes aspartyl protease abundantly. Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Most aspartic proteases show maximal activity at low pH (pH 3 to 4), aspartic protease in *Rhizopus microsporus var. oligosporus* is pepsin-like enzymes (Rao, Tanksale, Ghatge, & Deshpande, 1998). Another enzyme that is secreted by *Rhizopus microsporus var. oligosporus* is serine protease, it digests only proteins with relatively small amino acid side chains, such as glycine or alanine, can be cut by this enzyme. Serine protease is generally active at neutral or alkaline pH, with an optimum activity between pH 7 and 11. In the commerical production, the mixed culture is inoculated through cooked soybean. There are some moulds, yeasts, and microfloras grow during fermentation, with *Rhizopus* as the dominant genus. Those species secrets particular enzyme to occur fermentation (Samson, 1987).

Umami is one of the basic tastes that can be detected by humans. Umami taste could be formed by free amino acid aspartic acid and glutamic acid (Qin & Ding, 2007), small peptide, that has molecular weight lowers than 3 kDa and contained aspartic acid or glutamic acid as results of protein digestion through fermentation (Zhang, Klebansky, Fine, Xu, Pronin, Liu, et al., 2008) nucleotide such as, ionosine monophospate (IMP) and guanosine monophospate (GMP) (Yamaguchi & Ninomiya, 2000). Umami taste receptor is a class C G-protein coupled receptor (GPCR), which responds to L-glutamate and, to some extent, L-aspartate. The receptor belongs to the T1R family, composed of the T1R1 and T1R3 members. It may function as as a heterodimer or a homodimer. T1R family taste receptors are related to the metabotropic glutamate receptors (mGluR).

Umami peptides were isolated from water soluble extracts from protein based product, they were purified using RP-HPLC and the amino acid sequences were identified using LC-MS/MS. In order to study the taste characteristic of the peptides, the sensory evaluation for the synthetic peptide is suggested (Su, Cui, Zheng, Yang, Ren, & Zhao, 2012). Currently, interactions between umami peptides and the umami receptor have not been explored yet. However, other umami taste agents have been evaluated such as L-glutamate (Lopez Cascales, Oliveira Costa, de Groot, & Walters, 2010) and guanosine monophosphate (Zhang, et al., 2008), and the interaction between the particular umami substances and the umami receptor was identified using molecular docking with homology models of the structure of T1R1/T1R3.

1.2. Research Objectives

This research was designed to

1. Identify the peptide sequence that contributed to umami taste using LC-MS/MS
2. Evaluate the taste profile of umami peptide using sensory evaluation
3. Explore the mechanism of the binding of umami peptide to umami receptor using molecular docking

1.3. Research Motivation

Tempeh plays an important role for the Indonesian food security, and it supplies much nutrition for humans. Tempeh has the highest consumption percentage, compared to other soybean-based products. Tempeh could be claimed as a significant contribution to

world heritage from Indonesia, and therefore is is worthwhile to explore the competitive advantages of tempeh. The tempeh research is expected to provide a good benefit to Indonesian people.

II. Review of Literature

2.1. Tempeh

Tempeh is a soybean product produced by fermentation (**Fig 1**). Tempeh is produced by several steps, including dehulling, acidification, boiling, colling, inoculation, and incubation. The acidification could be conducted by some different scenarios: they are soaking the dehulled soybean into acid water contains particular acid compounds, such as lactic acid, citric acid, or acetic acid at pH 5 (Baumann & Bisping, 1995), inoculating the dehulled soybean using pure culture of *Lactobacillus sp* or soaking dehulled soybeans into tap water overnight, which allows the lactic acid bacteria to grow and produce lactic acid during acidification. All of the acidification stimulates the decrease of soybean pH (Mo, Kariluoto, Piironen, Zhu, Sanders, Vincken, et al., 2013). Acidification is used to enhance the efficiency of fermentation, since the low pH induces the growth of moulds and enhances protease activity during fermentation.

Fig 1. Tempeh (fermented soybean based product)

In traditional and practical tempeh production in Indonesia, the starter culture is usually prepared from sporulating tempeh of a previous fermentation and therefore consists of a mixture of molds, yeasts, and bacteria, with *Rhizopus* as the dominant genus.

However, the use of pure culture is more recommended related to its safety for human consumption (Samson, 1987).

One of the moulds that is responsible during tempeh fermentation is *Rhizopus microsporus var. oligosporus,* which secretes protease to digest the soy protein into some small peptides and free amino acids (Ismail, 1981). The higher amount of macronutrient in cooked soybeans (**Table 1**) stimulates the growth of *Rhizopus* spores and its enzyme secretion during fermentation.

Fungal growth and enzymatic activity are essential for an appropriate quality of product formation. The temperature and pH for *Rhizopus microsporus var. oligosporus* are 42 °C and 5 respectively. In this phase of germ protrusion, the presence of exogenous carbon and nitrogen is required. *Rhizopus microsporus var. oligosporus* grows faster than most moulds and quickly colonizes the substrate. The consideration of using *Rhizopus microsporus var. oligosporus* as starters for tempeh fermentation are the rapid growth, high lipolytic activity, strong antioxidant activity, inability to hydrolyze sucrose, its ability to produce the typical tempeh flavor, aroma, and texture, and high proteolytic activity. *Rhizopus microsporus var. oligosporus* could inhibit the grow of bacteria and alfatoxin production during tempeh fermentation. Currently, there is no information reported on the alfatoxin contamination during tempeh fermentation and its final product.

Table 1. Nutrition facts in 100 gr cooked soybean

Nutrition	Amount (gr)
Total carbohydrate	10
Total Fat	9
Protein	17
Sodium	0.001

Source : USDA SR-21(2014)

The lag phase of *Rhizopus microsporus var. oligosporus* during tempeh processing depends on the spore viability temperature, concentration of undissociated organic acids, and pH. By increasing the initial temperature and the optimal concentration of organic acid, a shorter lag phase can be obtained. Pure culture fermentation is necessary for industrial tempeh manufacture.

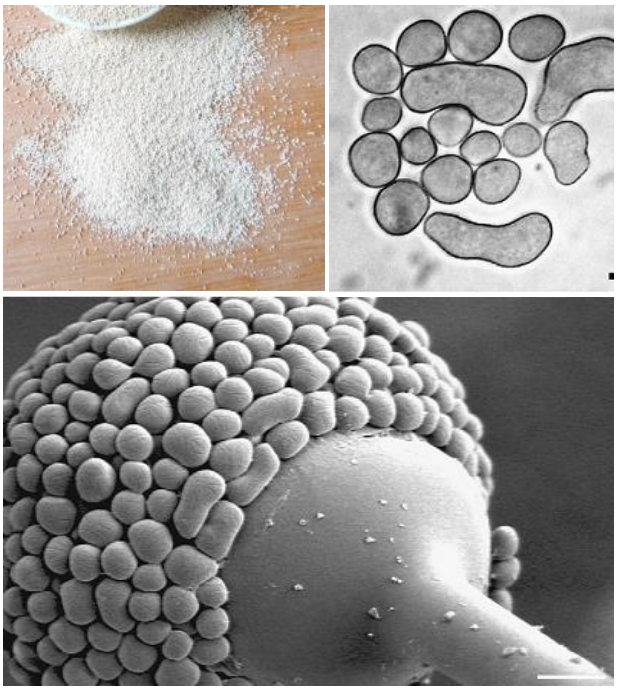

Fig 2. *Rhizopus microsporus var. oligosporus*
Source : Jennessen, Schnurer, Olsson, Samson, and Dijksterhuis (2008)

2.2. Proteolysis during tempeh fermentation

The fungus *Rhizopus microsporus var. oligosporus*, a class of Zygomycetes, is traditionally used to make tempeh, which was originated from most parts of Indonesia archipelago. Its roles are protein, carbohydrate and lipid digestion during tempeh fermentation. The digestion of macronutrients during fermentation could occur because of the secretion of protease, lipase, and alpha amylase during fermentation (**Table 2**).

Table 2. Enzymes produced by *Rhizopus microsporus var. oligosporus* (*R. Microsporus var.oligosporus*) by the following production factor relative humidity (%), temperature (ºC), incubation time (h) were 95-97, 30, and 48 respectively.

Enzyme	Activity (U/g)
Protease	97.9
Lipase	177.0
Glutaminase	170
α- amylase	257

Source : Han, Ma, Rombouts, and Robert Nout (2003)

Aspartic acid protease is commonly known as acidic protease. It is an endopeptidase that depends on aspartic acid residues for its catalytic activity. Aspartic protease shows

maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. The behaviour of intracellular and extracellular proteases was similar in relation to pH, temperature and protease inhibitors. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Aspartic acid proteases from *Rhizopus sp.* is pepsin-like protease (Rao, Tanksale, Ghatge, & Deshpande, 1998). Optimal temperature for the protease systems was found to be 55°C for *Rhizopus microsporus var. oligosporus* and the highest protease activities were found after fermentation times of 45-70 hours (Baumann & Bisping, 1995).

2.3. Umami taste

Umami is one of five basic tastes that could be recognized by humans. Umami taste could be intensified by free amino acid aspartic acid or glutamic acid (Qin and Ding, 2007), as well as by small peptides with molecular weight <3 kDa which result from protein digestion, and which contain aspartic acid or glutamic acid residues (Gómez-Ruiz, Taborda, Amigo, Ramos, & Molina, 2007; Maehashi, Matsuzaki, Yamamoto, & Udaka, 1999; Su, Cui, Zheng, Yang, Ren, & Zhao, 2012; Zhang, Wang, Liu, Xu, & Zhou, 2012), and nucleotides such as ionosine monophospate (IMP) and guanosine monophospate (GMP) (Yamaguchi & Ninomiya, 2000). Previously reports have demonstrated synergistic effect of NaCl and monosodium glutamate on peptides (Wang, Maga, & Bechtel, 1996).

Table 3. List of umami peptide sequences, their characteristics and their sources.

No	Source	Treatment	Peptide sequence	MW(kDa)
1	Peanut	enzymatic	SSRDEQSR	<3
2	Manchego Cheese	fermentation	EQEEK, QEEK, EINEK	<1
3	Puffer fish	untreated	YGGTPPFV	<3
4	Cheese	fermentation	EE, EV, ED, ADE, AED, DEE, SPG, EEN, LSERYPDADV,	<3
5	Chicken	fermentation	DA,DV,EE,EV,ADE,AED, DEE,DES,EEN,SPE,EPAD	<1
6	Beef	untreated	KGAEESLA	<3

2.4. The mechanism of binding umami to receptor

The T1R1/T1R3 heterodimer is coupled to a heteromeric G-protein, where the $G_{\beta\gamma}$ subunit appears to mediate the pre dominant leg of the signaling pathway. The complex of T1R-ligand activates $G_{\beta3\gamma13}$ and results in activation of phospholipase $C_{\beta2}$ ($PLC_{\beta2}$) (Huang, Shanker, Dubauskaite, Zheng, Yan, Rosenzweig, et al., 1999), which produces inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates the type III IP_3 receptor (IP_3R3) (Clapp, Stone, Margolskee, & Kinnamon, 2001), which results in the release of Ca^{2+} from intracellular stores and Ca^{2+} dependent activation of TRPM5 (Pérez, Huang, Rong, Kozak, Preuss, Zhang, et al., 2002). TRPM5 is thought to depolarize taste cells, which results in action potential generation and release of ATP, which activates ionotropic purinergic receptors on gustatory afferent nerve fibers (Finger, Danilova, Barrows, Bartel, Vigers, Stone, et al., 2005).

Umami taste transduction was reviewed by some researches. First, all of these signaling effectors are co-localized with the receptor T1R1/T1R3. Second, knockout of $PLC_{\beta2}$, IP_3R3, and TRPM5 all reduce umami taste responses in a manner similar to that of the knockout of T1R3. Third, pharmacologic inhibitors of $PLC_{\beta2}$ and Ca^{2+} ATPase, which maintain intracellular Ca^{2+} stores, virtually eliminate responses to glutamate and nucleotides applied selectively to the taste pores in Ca^{2+} imaging studies of a lingual slice preparation (Maruyama, Pereira, Margolskee, Chaudhari, & Roper, 2006)

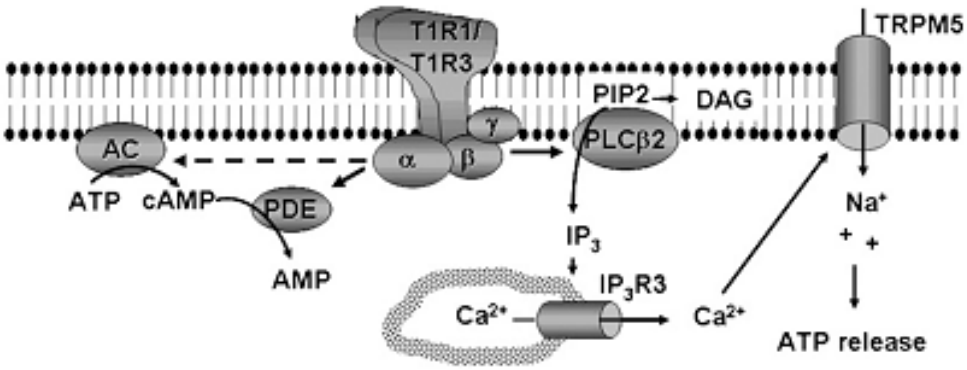

Fig 3. Model illustrating the signaling effectors downstream of the umami receptor T1R1/T1R3 (Kinnamon, 2009)

The G_{α} subunit mediates umami transduction based on tasted field. T1R1/T1R3 co-localized with α-gustducin in fungiform and palatal taste buds and undentified G_{α} in circumvallate and foliate taste buds (Kim, Kusakabe, Miura, Shindo, Ninomiya, & Hino, 2003). G_{α}-gustducin is related to G_{α}-transducin, which is also expressed in taste buds. Both α-gustducin and α-transducin activate phosphodiesterases (PDEs), which results in decreases in intracellular cAMP concentrations. Knockout of either α-gustducin or α-transducin compromises umami taste, which suggests that both G_{α}-gustducin and G_{α}-transducin participate in umami transduction. Physiologic studies also support a role of cAMP in umami taste. Because the activation of PDEs suppresses cAMP concentrations, cAMP should antagonize responses to umami stimuli.

III. Material and Method

3.1. Material

Soybean was purchased commercially in a traditional market in Neipu, Pingtung, ROC, commercial tempeh mould was purchased in home industry in Malang, Indonesia, Acetonitrile, TFA, citric acid, caffein, sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, USA), sodium chloride was purchased from Taiyen Biotech Co., LTD. (Miaoli, Taiwan), monosodium glutamate was purchased from AJINOMOTO (Bangkok, Thailand), and Formic acid (FA) was purchased from J. T. Baker (Phillipsburg, NJ, USA). Molecular weight cut-off (MWCO) ultra-filtration membranes with a 3 kDa cut-off were procured from Millipore (Bedford, MA, USA). The synthetic peptide (GENEEEDSGAIVTVK) was obtained from MDBio, Inc. (Taipei, Taiwan). The water used in this study was obtained using a Milli-Q® water purification system from Millipore (Billerica, MA, USA). All other chemicals used were of analytical grade.

3.2. Tempeh production and water soluble extract (WSE) preparation

Soybean was dehulled, then soaked in acid water, which contained citric acid (pH : 5) for 6 h, then boiled, and cooled before inoculation. 0.2% (w/w) inoculum (*Rhizopus microsporus var. oligosporus*) was mixed through boiled soybean. Tempeh was fermented in a plastic polyethylen with 5 holes in each pack; the incubation was performed in an incubator at 30ºC for 48 h. Tempeh was pulverized with mortar and pestle and throughly dried before use, then milled to powder.

Dried tempeh was diluted in water by the following proportion (tempeh : water = 1:4), heated at 40 ºC for 1 hour. The mixture was extracted (3.600 g; 20ºC, 30 min), the supernatant was reextracted using ultracentrifuge with following condition (10.000 g; 20ºC; 30 min). The supernatant was taken and dried (Gómez-Ruiz, Taborda, Amigo, Ramos, & Molina, 2007)

3.3. Collection of small peptides using 3 kDa cutoff membrane

Dried extract of tempeh was pulverized and dissolved in water, passed through ultrafiltration membrane in a ultracentrifuge (14.000 g; 40 min, 20ºC). The filtrate was

lyophilized before use for analysis (Gómez-Ruiz, Taborda, Amigo, Ramos, & Molina, 2007)

3.4. Peptide identification using ESI LC-MS/MS

Freeze dried < 3kDa cutoff fraction was dissolved in 5% ACN and 0.1% FA in deionized water for LC–MS/MS analysis. LC–MS/MS analysis was performed using a Thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source (Thermo Scientific Inc., USA). Samples were loaded onto C18 column (d : 150 × 2.1 mm, particle siz : 5μm). The samples were eluted using a gradient from 5 % to 70% acetonitrile in 0.1% formic acid over 75 min. Mass spectral data was detected using Thermo Xcalibur™data acquisition. The sheath gas flow rate was 50 arb, spray voltage applied for full mass scan was 4 kV and the capillary voltage was 20 V with capillary temperature of 300 °C. MS scan from m/z 100 to m/z 1000 was performed, with a flow rate of 200μl/min. The MS/MS raw data were obtained using Thermo-XCalibur™(Thermo-Scientific) then processed into MGF files using Mascot Distiller v 2.3.2.0 (Matrix Science, London, UK). The resulting MGF files were searched using the Mascot search engine v 2.3 (Matrix Science, UK) with the following search parameters: 1. protein database was set to be a home-made database 'Soybean' which was established from the combined Fasta files of soybean; 2. the enzyme was set as 'none enzyme'; 3. the precursor and product ion mass tolerance was set at 2 Da/1 Da; 4. the significance threshold was $p < 0.05$. The peptide sequence was identified through database matching as well as the manual interpretation of its MS/MS spectrum. Peptides with ion scores more than the identity threshold (score > 65) were regarded as identified peptides. The identified peptide sequence was persued by comparing the retention time of synthetic peptide based on the retention time, m/z, and MS/MS spectra with identified peptide in sample (Rawendra, Aisha, Chang, Aulanni'am, Chen, Huang, et al., 2013). The enzyme that contributed to protein digestion in this study was mapped using proteomics tools (peptide cutter) on the ExPASy molecular biology server http://web.expasy.org

3.5. Sensory evaluation

The sensory evaluation used a protocol based on that followed by Su, Cui, Zheng, Yang, Ren, and Zhao (2012). Panelists were chosen from Department of Biological

Science and Technology, National Pingtung University of Science and Technology, and the panelists age was 23-26 years old. The panelists were trained to recognize the five basic tastes, including sweet, bitter, sour, salty, and umami.

Taste profiles for dried water soluble extract from tempeh were analysed using a 10 point intensity scale (1, no taste; 10, very strong taste). Taste reference samples for sweet, bitter, sour, salty and umami were solutions of sucrose (1%), caffeine (0.08%), citric acid (0.08%), sodium chloride (0.35%), and MSG–salt (0.35–0.35%), respectively.

Descriptive analysis was performed to determine the differences of taste characteristics between water soluble extract and < 3 kDa cutoff fraction, < 3 kDa cutoff fraction and synthetic peptide GENEEEDSGAIVTVK, respectively. 1 mg dried water soluble extract and 1 mg < 3 kDa cutoff fraction were tested, five sensory attributes of < 3 kDa cutoff fraction weres evaluated and water soluble extract was selected as standard and each attribute was ranked 10 when used as the standard. Then, 1 mg synthetic peptide and 1 mg < 3 kDa cutoff fraction, were tasted, umami taste of < 3 kDa cutoff fraction was selected as control and ranked 10, five sensory attributes of synthetic peptide GENEEEDSGAIVTVK were evaluated and < 3 kDa cutoff fraction was selected as standard and ranked 10.

3.6. Statistical analysis

Statistical calculation was performed by the statistical package SPSS 22.0 (IBM SPSS Statistics 22) for one-way ANOVA. The Student–Newman–Keuls (S-N-K) test was used for comparison of mean values among treatments, and to identify significant differences ($p < 0.05$) among treatments.

3.7. **Molecular docking of identified peptide to umami receptor**

The protein receptor is a heterodimer T1R1/T1R3, and the best available structure was established by homology modeling performed by Lopez Cascales, Oliveira Costa, de Groot, and Walters (2010) (no X-ray structure is available). The homology modeling was carried out using the closed-open state of mGluR1 as the template (PDB code 1EWK). The ligand binding domain of the mGluR1 has 26.8% sequence identity with human T1R1 and 24.1% identity with human T1R3. Two models were generated for the umami receptor, including form 1 which has T1R1 in the closed conformation and T1R3 in the open

conformation, and form 2 which has T1R1 open and T1R3 closed. The heterodimer T1R1/T1R3 for each form was separated into the homodimer T1R1 and T1R3 before simulation. Therefore, there were four protein conformations available as input protien structures for molecular docking (**Fig 4**).

The preparation of peptide GENEEEDSGAIVTVK was done by submitting to protein modeling web server (http://ps2v2.life.nctu.edu.tw/), a knowledge based method to build peptide conformations. For each conformation of peptide GENEEEDSGAIVTVK the electrostatic solvation energy was calculated using CHARMm GUI PBEQ-Solver web server (Jo, Vargyas, Vasko-Szedlar, Roux, & Im, 2008). Those conformations were visualized using RASMOL version 2.7.2.5.

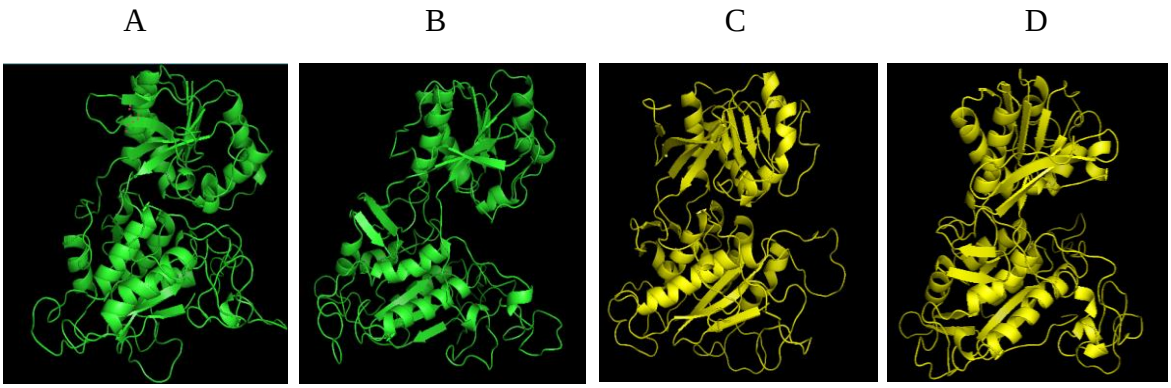

Fig 4. T1R model, separated from heterodimer T1R1/T1R3; A. T1R1 in closed conformation; B. T1R1 in open conformation; C. T1R3 in closed conformation; D. T1R3 in open conformation.

The molecular docking was performed using an online docking server (http://zdock.umassmed.edu/), and the binding site for each receptor proteins conformation was adjusted according to Lopez Cascales, Oliveira Costa, de Groot, and Walters (2010). The amino acid position in the reference were matched with the amino acids position in current models before adjusting the binding pocket in the model, because the homodimer T1R1 and T1R3 were separated from the heterodimer T1R1/T1R3, and amino acid renumbering was needed. The binding site for each of the current models are given in **Table 4**. This system was confirmed by docking the L-Glu (glutamic acid) into each protein receptor conformation in its adjusted binding pocket.

Table 4. The amino acids residue in each receptor conformation

T1R1 in reference	T1R1 closed	T1R1 open	T1R3 in reference	T1R3 closed	T1R3 open
Ser 148	121 Ser	122 Ser	His 145	577 His	2814 His
Thr 149	122 Thr	123 Thr	Ser 147	579 Ser	2816 Ser
Arg 151	124 Arg	125 Arg	Gly 168	600 Gly	2837 Gly
Ala 170	141 Ser	142 Ser	Ser 170	602 Ser	2839 Ser
Ser 172	143 Ala	144 Ala			
Arg 277	250 Arg	251 Arg			
Glu 301	274 Glu	275 Glu			

The best conformation from ZDOCK was selected, minimized using CHARMm and then its ΔG was calculated. The interaction between the binding pocket in the receptor and peptide GENEEEDSGAIVTVK was visualized using PYMOL version 1.7.0.3, and Accelrys Discovery Studio version 4.0.

IV. Result and Discussion

4.1. Identification of umami peptide in tempeh using ESI LC-MS/MS

LC-MS/MS has been commonly used to discover compounds' structure and molecular weight. Some novel compounds have been discovered by using this method. In this case, the dried water soluble extracts from tempeh were diluted in water and passed through 3 MWCO membrane for ultrafiltration, so therefore the size of filtrate is less than 3 kD. The < 3 kDa cutoff fraction was diluted in 5% ACN and 0.1% FA in deionized water and loaded to LC-MS/MS for peptide identification. This method was used by Gu and Wu (2013) to discover some novel angiotensin I converting enzyme inhibitor peptides from crude extract of soy protein hydrolysate. Umami peptides from protein hydrolysate have been discovered, including those having some glutamic acid or asparatic acid sequence (Gómez-Ruiz, Taborda, Amigo, Ramos, & Molina, 2007a; Maehashi, Matsuzaki, Yamamoto, & Udaka, 1999; Rhyu & Kim, 2011; G. Su, Cui, Zheng, Yang, Ren, & Zhao, 2012; M. X. Zhang, Wang, Liu, Xu, & Zhou, 2012), and therefore this study was focused to identify peptides with glutamic acid or asparatic acid sequence, which resulted from mascot search, and the amino acid sequence with more glutamic acid would be assessed as the contributor of umami taste (**Table 5**).

The m/z and molecular weight of the identified peptide is 789.63 and 1577.25 Da, respectively, which was shown by mascot search, then the mass spectra was analyzed by using de Novo sequencing, based on its *y* and *b* ion position (**Fig 5**). The reproducibility of this method was confirmed using synthetic peptide GENEEEDSGAIVTVK, by comparing its m/z and retention time with experimental data, and the results showed the high similarity between experimental and synthetic peptide (**Table 6**). This peptide is supposed to be a hydrophilic peptide, because it could be eluted quickly in LC-MS/MS. According to calculations using mascot search, the protein origin of this peptide is glycinin subunit G2 [Glycine max], glycinin is major soy proteins, it presences at the proportion greater than 85 % (Keshun, 1997)

Table 5. Identified peptide from < 3 kDa cutoff fraction of tempeh water soluble extract

Identified protein	Identified peptide	Position Start–end	m/z	Mr	Mascot score
Glycinin subunit G2	GENEEEDSGAIV TVK	242-256	789.64	1577.25	104
Alpha' subunit of beta-conglycinin	DEGEQPRPFPFP	33-44	708.89	1414.65	85
Mutant glycinin subunit A1aB1b	GENEGEDKGAIV TVK	245 - 259	773.99	1544.77	70
	NLQGENEGEDK GAIVTVK	242 - 259	951.16	1899.95	78

Table 6. Comparison of t_r and m/z identified peptide in < 3 kDa cutoff fraction and synthetic peptide

Peptide	t_R	m/z
Identified peptide from < 3 kDa cutoff fraction	10.72	789.64
Synthetic peptide	9.39	789.08

The peptide cutter in the ExPASy Molecular Biology Server (http://kr.exp-asy.org/) was used to search the protease, which could produce peptide GENEEEDSGAIVTVK. The pattern of protein digestion was modeled from the origin sequence, according to (Keil, 1992) (**Fig 6**). The protease in peptide cutter was adjusted and mapped into glycinin subunit G2, based on cleavage site. From calculations using the Proteomics tools (Peptide cutter) on the ExPASy Molecular Biology Server, there is no spesific protease cleavages this particular position. A similar phenomena was found by (G. Su, Cui, Zheng, Yang, Ren, & Zhao, 2012; Guowan Su, Ren, Yang, Cui, & Zhao, 2011), while that work used *Aspergillus oryzae* to digest defatted peanut, the results showed that no sequence match, because of *Aspergillus oryzae* could secretly a complex array enzyme, such as protease, lipases, and cellulolytic enzyme, which it could hydrolyze the peanut protein to peptides simultaneously, and make the free amino acid bond to peptides. In this work, the mould utilized nutritions in cooked soybean and secret some enzymes to digest soyprotein to peptides. During the growth of mould miselium, protease, lipases, α-amylase, and glutaminase were secreted (Han, Ma, Rombouts, & Robert Nout, 2003) and its may digest soy proteins to peptides simultanously.

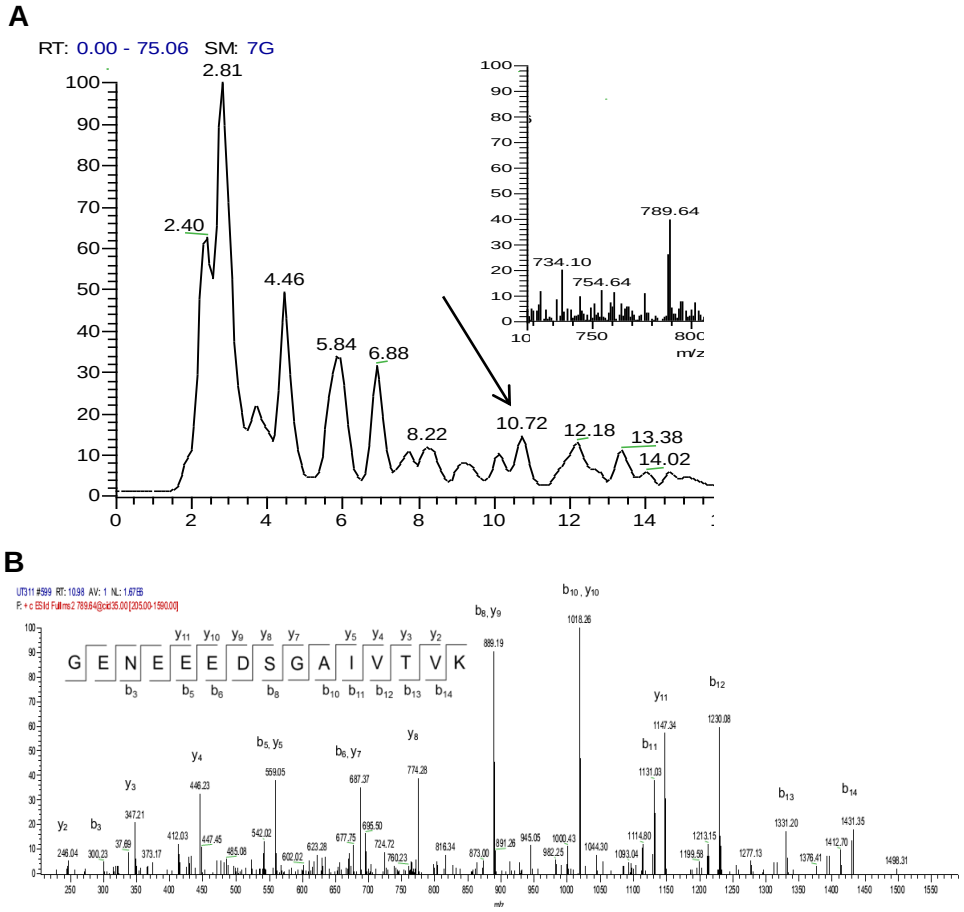

Fig 5. A. LC–MS chromatogram of 3 kDa fraction The inset **Fig**ure shows the precursor ion of the at m/z 789.64; **B**. ESI-LC-MS/MS spectrum of precusor ion m/z 789.64

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    10         20         30         40         50         60
MAKLVLSLCF LLFSGCFALR EQAQQNECQI QKLNALKPDN RIESEGGFIE TWNPNNKPFQ

    70         80         90        100        110        120
CAGVALSRCT LNRNALRRPS YTNGPQEIYI QQQGNGIFGMI FPGCPSTYQE PQESQQRGRS

   130        140        150        160        170        180
QRPQDRHQKV HRFREGDLIA VPTGVAWWMY NNEDTPVVAV SIIDTNSLEN QLDQMPRRFY

   190        200        210        220        230        240
LAGNQEQEFL KYQQQQQGGS QSQKGKQQEE ENEGSNILSG FAPEFLKEAF GVNMQIVRNL

   250        260        270        280        290        300
QGENEEEDSG AIVTVKGGLR VTAPAMRKPQ QEEDDDDEEE QPQCVETDKG CQRQSKRSRN

   310        320        330        340        350        360
GIDETICTMR LRQNIGQNSS PDIYNPQAGS ITTATSLDFP ALWLLKLSAQ YGSLRKNAMF

   370        380        390        400        410        420
VPHYTLNANS IIYALNGRAL VQVVNCNGER VFDGELQEGG VLIVPQNFAV AAKSQSDNFE

   430        440        450        460        470        480
YVSFKTNDRP SIGNLAGANS LLNALPEEVI QHTFNLKSQQ ARQVKNNNPF SFLVPPQESQ
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Fig 6. Mapping of protein digestion in tempeh production from Glycinin subunit G2, the arrow represents starting position (P_1^1) and the red font represents amino acid position P_1^1 to P_{15}^1.

4.2. Sensory evaluation of umami taste in tempeh

According to Maehashi, Matsuzaki, Yamamoto, and Udaka (1999), the sensory properties of proteins can be increased through hydrolysis by certain enzymes. In this study, five basic tastes of water soluble extract were evaluated, five basic tastes were recognized by panelists, which described in **Fig 7**. The results showed that bitter taste had the highest rating (6.83) among five basic tastes, followed by umami and salty, which the rating are 5.50 and 5.17 respectively, while sweet and sour were evaluated with rather low scores (**Fig 7**). Five of basic tastes intensities in tempeh hydrolysate were significant different to standar taste agents ($p<0.05$) and generally the five basic tastes in tempeh hydrolysate were lower than the standard for basic taste.

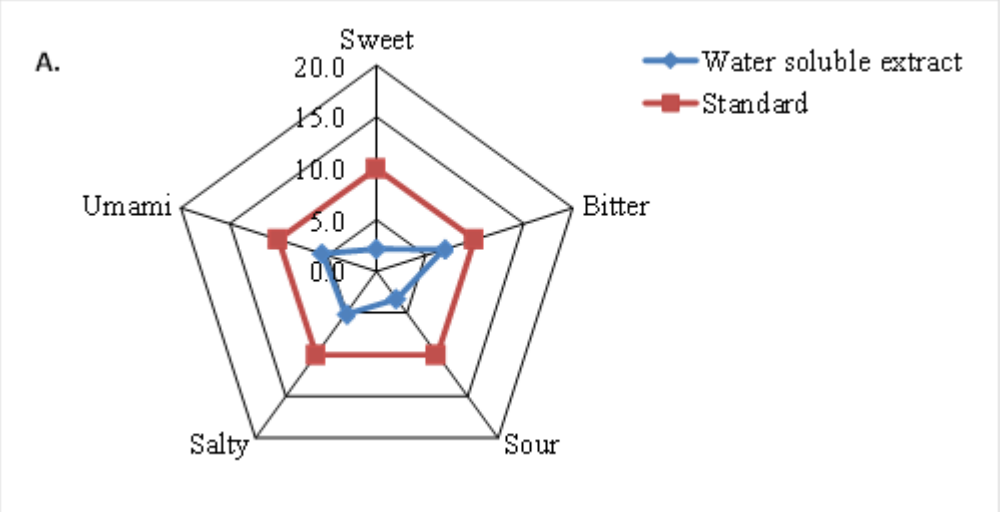

Fig 7. Taste profile of water soluble extract from tempeh

In order to identify the key compounds that contributed to intense tastes, the hydrolysate was used for further sensory guided fractionation to identify the taste. Due to umami taste was foccused on this study, ultrafiltration is used to get peptides, that have low molecular weight and high umami taste intensity. In this study, umami taste intensity of <3 kDa cutoff fraction was higher than water soluble extracts from tempeh ($p<0.05$) (**Fig 8**). This work was in agreement with the results of (G. Su, Cui, Zheng, Yang, Ren, & Zhao, 2012), while the protein hydrolyzate was fractioned to lower molecular weight, the umami taste was increased with decreasing of molecular weight.

In this work, ultrafiltration was stimulated with pressure driven in a centrifuge (Byun & Kim, 2001; Jang & Lee, 2005). During this process, the free amino acids, salt, organic acids, < 3 kDa cuoff peptide pass through filter membrane, then the free amino acids and salt may have synergistic effects on peptide, which could intensify more umami taste (Wang, Maga, & Bechtel, 1996). Therefore, the sensory evaluation for purify peptides must be conducted. In ultrafiltration, there are some of free amino acids and peptides with positive and negative charges appear in the large molecular weight fraction, due to its aggregation. On the other hand, some large molecular weight compounds, such as oligomers and polymers with high molecular weight, would interact with low molecular weight compounds and absorb them (Chevance & Farmer, 1999). These are could be a reason, that separation for precise molecular weight compound is hard to perform.

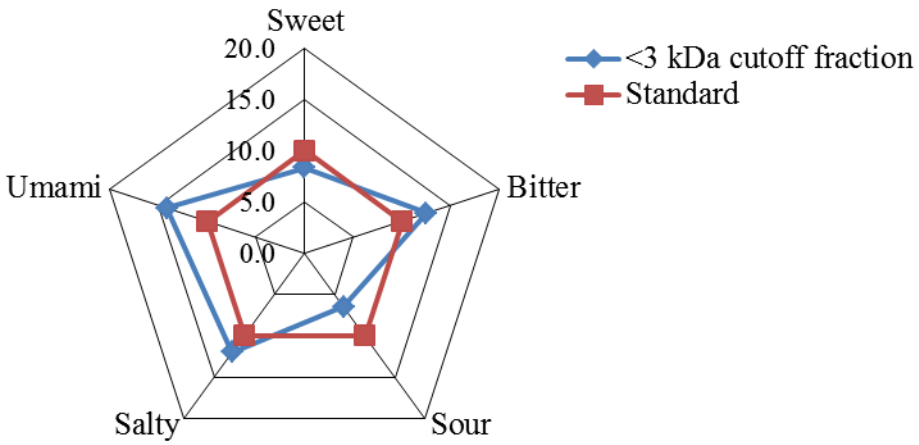

Fig 8. The difference taste attributes between water soluble extract (standard) and < 3kDa cutoff fraction.

Some previous researchers claimed that a peptide, containing Asp or Glu sequence could contribute to umami taste, therefore in this work peptide GENEEEDSGAIVTVK was used for taste profile evaluation. 95 % synthetic peptide was considered and it was described in **Fig 9**. This peptide elicited all of basic tastes, which umami has the highest intensity, followed by salty, bitter, sweet, and sour. The hydrophilic peptides were commonly associated with desirable flavours such as sweet, meathy, and brothy while the hydrophobic peptides were usually associated with more undesirable taste (Spanier & Edwards, 1987).

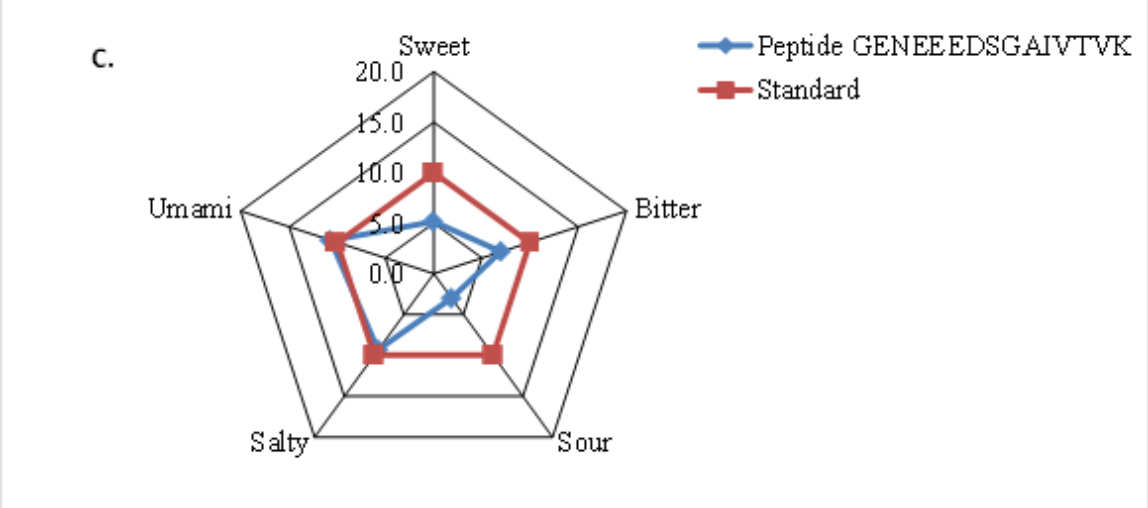

Fig 9. The difference taste attributes between 3 kDa fraction (standard) and peptide GENEEEDSGAIVTVK

Peptide GENEEEDSGAIVTVK could be seen has some hydrophilic amino acids such as glycin, glutamic acid, aspartic acid, and threonine, those amino acids evaluated as sweet taste eliciting amino acids, and those are considered to be the main contributor of sweet and umami taste (Lioe, Takara, & Yasuda, 2006), in the other hands the existing of some hydrophobic amino acids, such as valine, alanine, and isoleucine could form a hydrophobic surface predicted to face the receptor could reduce the sweetness **Fig 13** (Xue, Szczepankiewicz, Thulin, Linse, & Carey, 2009), and the existance of valine close to the C terminal may be a reason for eliciting the bitter taste by this peptide. The hydrophobic amino acids might play an important role to suppress the umami taste (Salles, Septier, Roudot-Algaron, Guillot, & Etievant, 1995). When the peptide was compared to ultrafiltration, the umami taste intensity in peptide was not significantly different from the <3 kDa cutoff fraction ($p>0.05$). Hence, this peptide may be one of several that contribute to the overall umami taste of the fraction.

4.3. Docking results for the interaction between umami peptide and receptor T1R

This work was designed to predict the binding poses possibile between umami peptide and each umami taste receptor conformation. It was started with umami peptide modeling, to predict its secondary structure. 25 GENEEEDSGAIVTVK conformations were generated by the ps2-v2 web server and each conformation has a different

electrostatic solvation energy (**Table 7)**, for which the least negative is predicted to be more stable in the protein environment. The lowest 10 conformations were considered for further analysis (**Fig 10**). The heterodimer of receptor protein for each form was separated using PYMOL before docking simulation. Previous research showed that umami substances bind into each dimer. Finally, there were 4 receptor conformations in this work including T1R1 in closed, T1R1 in open, T1R3 in closed, and T1R3 in open conformation.

Table 7. Solvation energy of 10 best peptide conformation, obtained from PBEQ solver CHARMm GUI

Rank	Conf	Solvation Energy (kcal/mol)
1	12	-904.65
2	4	-892.99
3	24	-891.19
4	22	-882.91
5	18	-881.79
6	23	-876.46
7	6	-875.62
8	20	-867.52
9	3	-864.45
10	7	-857.67

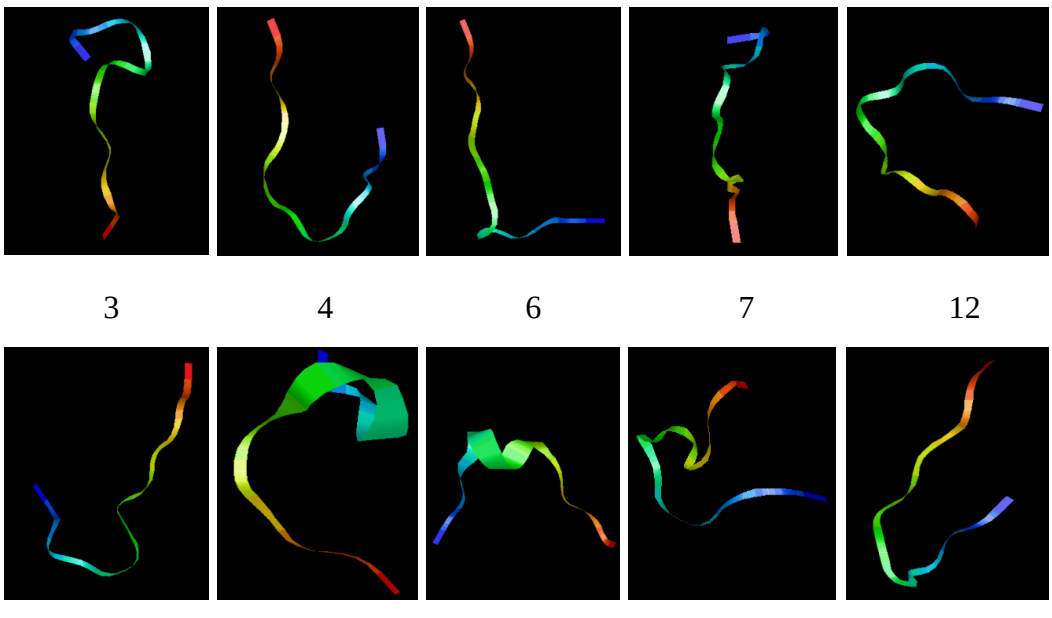

Fig 10. The lowest (most stable) 10 umami peptide conformations obtained from (PS)2-v2: Protein Structure Prediction Server and visualized using RASMOL 2.7.2.5.

The previous work performed the docking of L-Glu to T1R1/T1R3 and it showed a strong interaction. Therefore, in this work the system was confirmed by re-docking L-Glu to T1R, and then we analyzed the interaction and calculated the ΔG. The results showed that the amino acid residues for each protein receptor conformation have strong interaction with L-Glu. Hence, this system could be considered to predict the interaction between T1R and peptide GENEEEDSGAIVTVK.

Each receptor conformation and peptide GENEEEDSGAIVTVK conformation was submitted to the ZDOCK web server, so there were 40 combinations as complexes. Peptide GENEEEDSGAIVTVK should have interactions with amino acid residues in T1R like the interaction of T1R-L-Glu. Another parameter is that ΔG must be more negative. The more negative indicates the more favourable interaction.

The complex of T1R receptor and peptide GENEEEDSGAIVTVK that had the best interaction resulted in the ZDOCK scores ≤ 730 (Pierce, Wiehe, Hwang, Kim, Vreven, & Weng, 2014). The high ZDOCK score represents peptide GENEEEDSGAIVTVK binding on the surface of the protein or diffused away from the binding pocket. In T1R3, there were four peptide conformations that have favourable interaction with T1R3 in closed conformation and two peptide conformations with T1R3 in open conformation. All of the favourable interactions have low ZDOCK score in range ≤730 (**Table 8**). Regarding the ZDOCK results, protein T1R3 both in open and closed conformation is more favourable receptor for umami peptide GENEEEDSGAIVTVK. T1R3 in open conformation is more favourable than T1R3 in closed conformation, based on free binding energy calculation, for which the lower energy (more negative) is the more favourable interaction (**Table 10**). Therefore, the docking interactions support that the peptide GENEEEDSGAIVTVK may be the source of the umami taste in tempeh.

In T1R1, all of the peptide GENEEEDSGAIVTVK conformations bind to the surface of protein T1R1 both in open and closed conformation (**Fig 11)** and they also have high ZDOCK scores, so therefore T1R1 is not the favourable receptor for this peptide. The ΔG is much lower than that of L-Glu, because the amino acid residues in peptide GENEEEDSGAIVTVK interact with other amino residues not found in the binding pocket.

Table 8. ZDOCK score for umami receptor-umami peptide interaction

Rank	Conf	Solvation Energy (kcal/mol)	ZDOCK score			
			Form 1		Form 2	
			t1r closed	t1r3 open	t1r1 open	t1r3 close
1	12	-904.65	994.23	863.30	891.86	932.80
2	4	-892.99	891.70	963.12	958.90	1040.34
3	24	-891.19	922.88	987.61	1078.58	702.74
4	22	-882.91	1025.93	968.34	919.93	667.40
5	18	-881.79	918.75	683.97	1239.47	565.89
6	23	-876.46	936.70	969.30	873.34	729.48
7	6	-875.62	907.52	1019.79	997.92	978.25
8	20	-867.52	910.25	589.22	1017.88	1024.85
9	3	-864.45	916.54	1117.34	950.45	1082.22
10	7	-857.67	881.60	996.78	898.59	1085.71

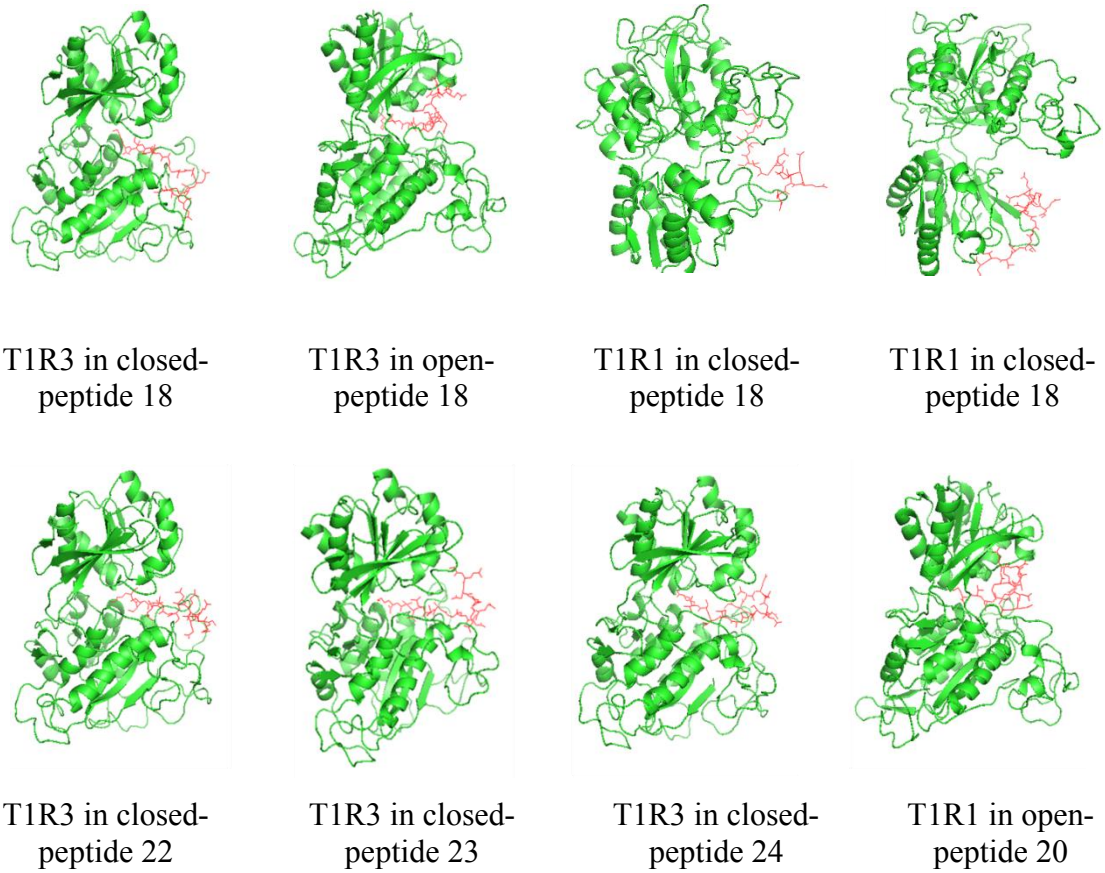

Fig 11. Complex of protein receptor T1R and peptide GENEEEDSGAIVTVK, obtained from ZDOCK web server.

L-Glu was selected as a control in this work, because it is considered a standard for umami taste and because its binding pose into protein receptor T1R1/T1R3it has been

previously studied, so then the docking results of T1R- peptide GENEEEDSGAIVTVK could be compared with those of L-Glu. In this work, peptide GENEEEDSGAIVTVK should have interactions with amino acid residues in T1R like the interaction of T1R-L-Glu. The ligand interactions analysis showed that peptide GENEEEDSGAIVTVK had some similar interactions with L-Glu in the binding pocket of T1R3 in open and closed conformation (**Fig 12**).

Most of the atoms of lysine (K) residue in peptide GENEEEDSGAIVTVK have similar interaction with those of L-Glu in the binding pocket T1R3, and glycine residue has one similar interaction with L-Glu in the binding pocket T1R3 in closed conformation. In T1R3 in open conformation valine, lysine, glutamic acid, and aspartic acid have one similar interaction with L-Glu in the binding pocket of T1R3 in open conformation.

According to molecular docking results, we can consider possible reasons why L-Glu may have more favourable umami taste than peptide GENEEEDSGAIVTVK: L-Glu could bind into the binding pocket of all T1R protein receptor conformations and it has strong interactions with more amino acid residues in the binding pocket. But, peptide GENEEEDSGAIVTVK only could bind into the binding pocket of protein receptor T1R3 in open and closed conformation. Although the ΔG for complex T1R–peptide GENEEEDSGAIVTVK is much more negative than T1R–L-Glu (**Table 10**), that is because the amino acid residues in peptide GENEEEDSGAIVTVK interacts with other non binding pocket amino residues in T1R1 and T1R3 in open/closed conformation (**Fig 12; Table 9**). The hydrophobic amino acid residues in peptide GENEEEDSGAIVTVK could form a hydrophobic surface predicted to face the receptor, which could reduce the savory taste (**Fig 13**) (Xue, Szczepankiewicz, Thulin, Linse, & Carey, 2009).

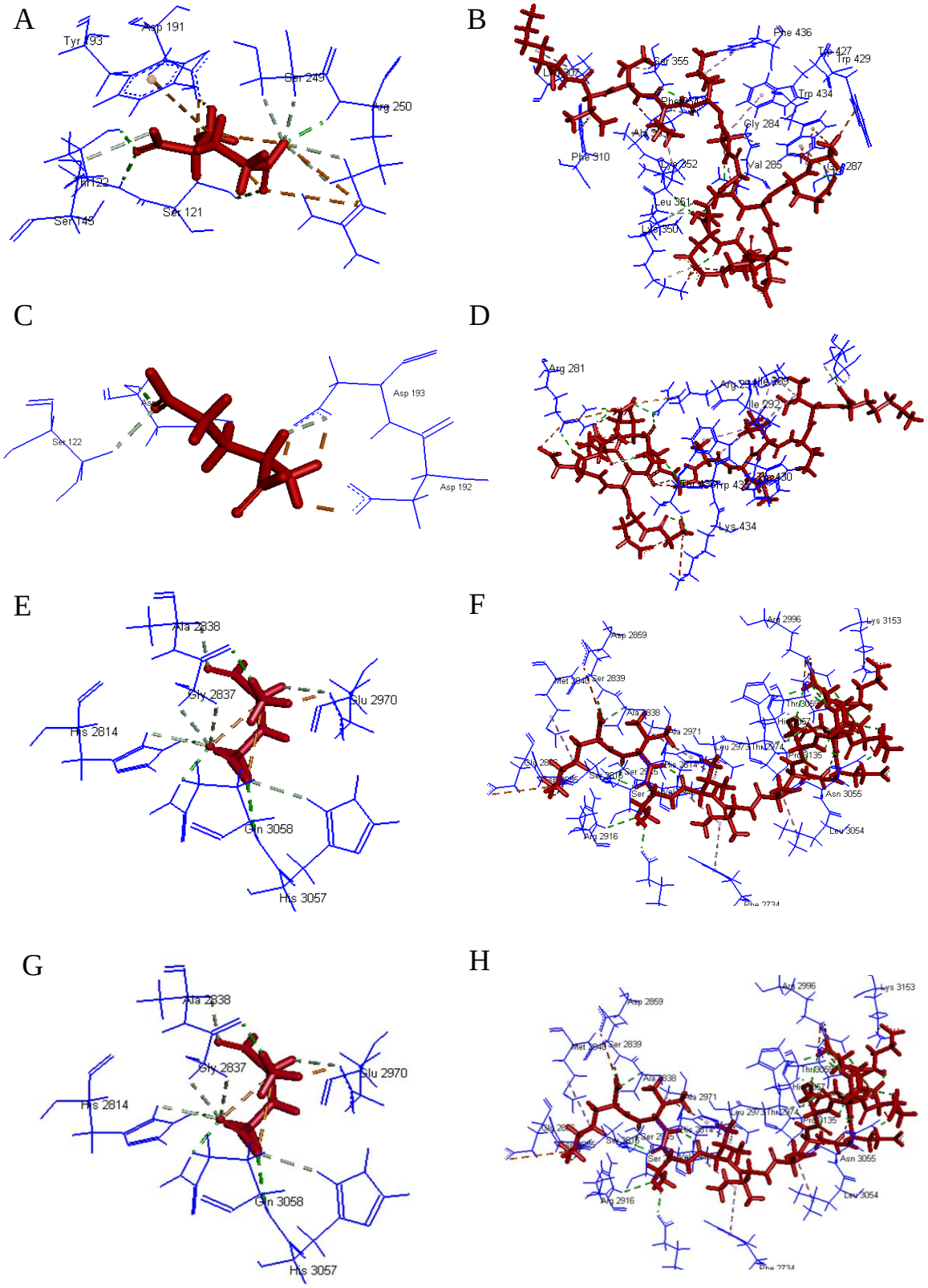

Fig 12. The receptor-ligand interactions, left (L-Glu), right (peptide GENEEEDSGAIVTVK) with binding pocket of T1R; A. T1R1 in closed; B. T1R1 in open; C. T1R3 in closed; D. T1R3 in closed

V. Conclusion

The water soluble extract of tempeh was fractionated using a <3 kDa molecular weight cutoff ultrafiltration membrane and the taste profile of the low molecular weight fraction was evaluated. The results showed that umami had the highest intensity compared to the other basic tastes. LC-MS/MS identified 4 peptides from the <3 kDa cutoff fraction, including GENEEEDSGAIVTVK, which we synthesized and evaluated. This peptide intensified umami taste. The results of molecular docking using ZDOCK showed that the peptide could bind to the binding pocket of TIR3 in open and closed conformation.

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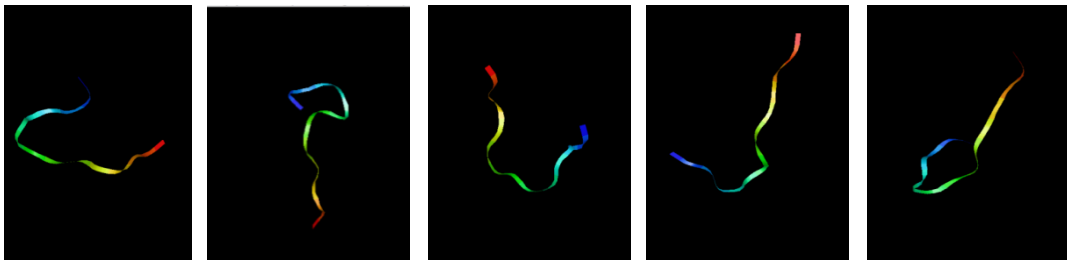
using an electronic tongue and MALDI-TOF/TOF MS/MS. *Food Chem*, *135*(3), 1463-1470.

Appendix I. Amino acid symbol and abbreviation

Amino acid	Symbol	Abbreviation
Alanine	A	Ala
Cysteine	C	Cys
Aspartic acid	D	Asp
Glutamic acid	E	Glu
Phenylalanine	F	Phe
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Lysine	K	Lys
Leucine	L	Leu
Methionine	M	Met
Asparagine	N	Asn
Proline	P	Pro
Glutamine	Q	Gln
Arginine	R	Arg
Serine	S	Ser
Threonine	T	Thr
Valine	V	Val
Tryptophan	W	Trp
Tyrosine	Y	Tyr

Appendix II.
Conformation number of peptide GENEEEDSGAIVTVK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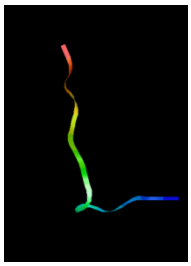
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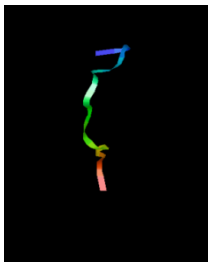
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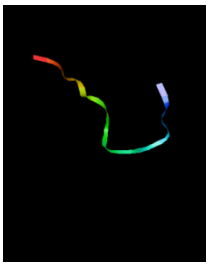
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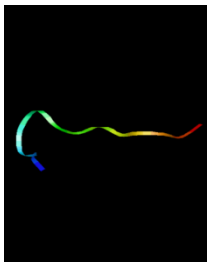
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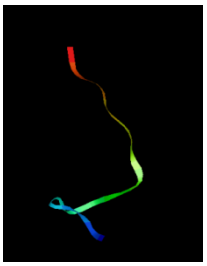
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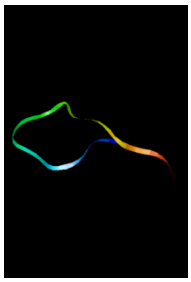
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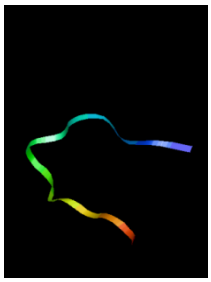
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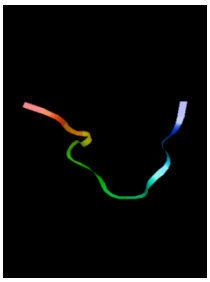
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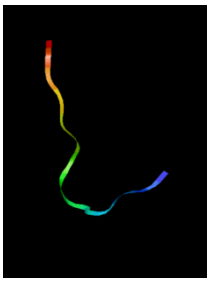
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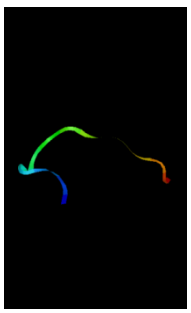
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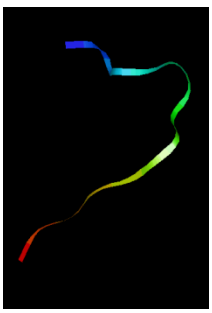
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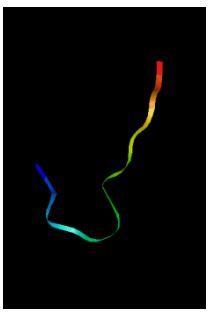
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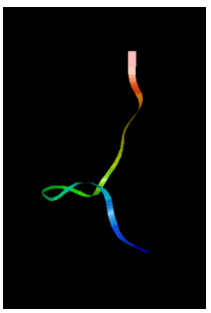
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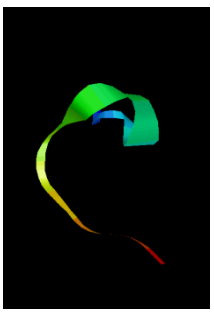
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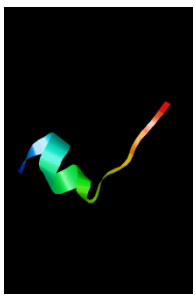
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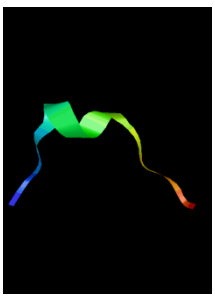
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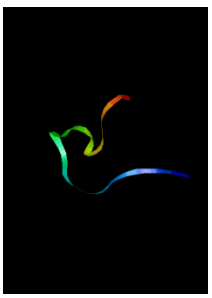
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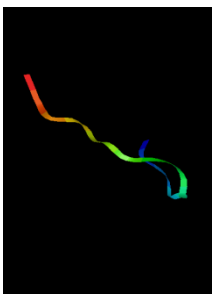
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Appendix III. Solvation energy of 25 peptide conformations, obtained from PBEQ solver CHARMm GUI

Conformation number	Solvation energy (kkac/mol)
1	-822.29
2	-821.79
3	-864.45
4	-892.99
5	-813.64
6	-875.62
7	-857.67
8	-802.23
9	-819.20
10	-843.22
11	-784.32
12	-904.65
13	-827.54
14	-792.44
15	-840.00
16	-814.51
17	-835.93
18	-881.79
19	-832.22
20	-867.52
21	-804.42
22	-882.91
23	-876.46
24	-891.19
25	-822.59

Appendix IV. ZDOCK results web server

T1R1 Closed

Conformation Rank	Conformation No	Website
1	12	http://zdock.umassmed.edu/results/690654697c/
2	4	http://zdock.umassmed.edu/results/da35c46b95/
3	24	http://zdock.umassmed.edu/results/229bf231a8/
4	22	http://zdock.umassmed.edu/results/f22beecfc7/
5	18	http://zdock.umassmed.edu/results/0368ac954b/
6	23	http://zdock.umassmed.edu/results/1c95d7df5d/
7	6	http://zdock.umassmed.edu/results/044b7206b2/
8	20	http://zdock.umassmed.edu/results/8540574e98/
9	3	http://zdock.umassmed.edu/results/662fd63dc8/
10	7	http://zdock.umassmed.edu/results/168e3f6917/

T1R3 Open

Conformation rank	Conformation No	Website
1	12	http://zdock.umassmed.edu/results/590b5209b1/
2	4	http://zdock.umassmed.edu/results/fc99d7e07c/
3	24	http://zdock.umassmed.edu/results/c189dbade9/
4	22	http://zdock.umassmed.edu/results/0e732ecae1/
5	18	http://zdock.umassmed.edu/results/bfd4e65d30/
6	23	http://zdock.umassmed.edu/results/6e2878c461/
7	6	http://zdock.umassmed.edu/results/b7a68f544c/
8	20	http://zdock.umassmed.edu/results/8a1cde2fd1/
9	3	http://zdock.umassmed.edu/results/798260cb75/
10	7	http://zdock.umassmed.edu/results/42838d51a3/

T1R1 Open

Conformation Rank	Conformation No	Website
1	12	http://zdock.umassmed.edu/results/7bc1660b5b/
2	4	http://zdock.umassmed.edu/results/a31c1ccdeb/
3	24	http://zdock.umassmed.edu/results/d6d2214bdc/
4	22	http://zdock.umassmed.edu/results/b8b9d19cfe/
5	18	http://zdock.umassmed.edu/results/5e371bd51f/
6	23	http://zdock.umassmed.edu/results/eaffa44c17/
7	6	http://zdock.umassmed.edu/results/ff613350ef/
8	20	http://zdock.umassmed.edu/results/43960800e1/
9	3	http://zdock.umassmed.edu/results/e673d8878d/
10	7	http://zdock.umassmed.edu/results/81ea233de6/

T1R3 Closed

Conformation Rank	Conformation No	Website
1	12	http://zdock.umassmed.edu/results/7d7b55a67a/
2	4	http://zdock.umassmed.edu/results/11fb7f90d5/
3	24	http://zdock.umassmed.edu/results/4bd8dc1aad/
4	22	http://zdock.umassmed.edu/results/c015546604/
5	18	http://zdock.umassmed.edu/results/56bdb01f3f/
6	23	http://zdock.umassmed.edu/results/1c30507889/
7	6	http://zdock.umassmed.edu/results/8d5b488552/
8	20	http://zdock.umassmed.edu/results/30d230f7a0/
9	3	http://zdock.umassmed.edu/results/ad4a4ab7aa/
10	7	http://zdock.umassmed.edu/results/79a25f64ad/

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